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RELATIONSHIPS BETWEEN FREE CUPRIC ION CONCENTRATIONS IN SEA WATER AND COPPER METABOLISM AND GROWTH IN CRAB LARVAE

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Abstract

Crab larvae (*Rhithropanopeus harrisii*) were exposed to a range of free cupric ion concentrations, $[Cu^{2+}]$, regulated in sea water by a metal chelate buffer system. We found a biphasic relationship between intracellular copper distribution and $[Cu^{2+}]$ in sea water. At $[Cu^{2+}]$ within the ambient range ($10^{-12.4}$ to $10^{-10.6}$ *M*), cytosolic copper was associated with both metallothionein (MT) and high molecular weight (HMW) ligands, and was independent of external $[Cu^{2+}]$. At higher $[Cu^{2+}]$, copper was also associated with very low molecular weight (VLMW) ligands, and accumulated in this ligand pool and the MT pool as external $[Cu^{2+}]$ increased. In marked contrast, copper in the HMW ligand pool did not correlate with $[Cu^{2+}]$ in sea water over the entire range of exposures. Reductions in larval growth occurred at greater than estimated ambient $[Cu^{2+}]$ and correlated with copper accumulation in the MT and VLMW pools.

Introduction

The concentrations and subcellular distributions of metals can provide valuable information on an organism's capacity to adapt to accumulated metals. As a consequence, it has been suggested that the more subtle aspects of metal toxicity in aquatic organisms can be more accurately estimated by examining the distribution of metals among the various intracellular ligand pools (Bayne *et al.*, 1980). However, to understand the ecological significance of these data we must also be able to relate this information on metal metabolism to effects on the organism, the population, and the community (Sanders *et al.*, 1983).

In a previous study on larvae of the crab *Rhithropanopeus harrisii* (Sanders *et al.*, 1983) we used metal-chelate buffer systems to control Cu speciation since the biological availability of Cu is related to the concentration of the free cupric ion, $[Cu^{2+}]$, rather than to the total or chelated Cu concentration (Sunda and Guillard, 1976; Anderson and Morel, 1978; Jackson and Morgan, 1978; Zamuda and Sunda, 1982). These buffers enabled us to expose larvae throughout zoeal development to a range of calculated $[Cu^{2+}]$ while the concentrations of potentially competitive metals were kept constant. In that study we found that most cytosolic Cu was associated with metallothionein, a cysteine-rich metal-binding protein whose synthesis is induced by metals (Hildebrand *et al.*, 1979). This ubiquitous protein has been implicated in metal uptake, metabolism, and detoxication in vertebrates and invertebrates (Richards and Cousins, 1976; Brown *et al.*, 1977; Li *et al.*, 1980; Roesijadi, 1980; Jenkins *et al.*, 1982). We found that the concentration of Cu-thionein in crab larvae was related

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to $[Cu^{2+}]$ in sea water, and an increase in Cu-thionein accumulation correlated with inhibition of larval growth.

In this study we have followed changes in high (HMW) and very low molecular weight (VLMW) Cu binding ligands as well as Cu-thionein. Also, we examined these ligand pools over a wider range of $[Cu^{2+}]$. We report relationships between $[Cu^{2+}]$, Cu-thionein, and larval growth which are similar to our previous findings. In addition, we found that the Cu concentration within the VLMW pool could also be related to the $[Cu^{2+}]$ in sea water, and that increases of Cu in this pool correlated with inhibition of larval growth. In contrast to a current hypothesis (Brown, 1977), we found no correlation between Cu associated with HMW ligands and either external $[Cu^{2+}]$ or inhibition of larval growth. Finally, we found that increases of Cu in the MT and VLMW pools, and inhibition of larval growth, occurred at $[Cu^{2+}]$ just beyond the estimated ambient range in the estuary where the crabs were collected.

Materials and Methods

Gravid females of the mud crab *Rhithropanopeus harrisii* were collected in the Newport River estuary near Beaufort, North Carolina. They were kept in tanks of running sea water and transferred to culture bowls when their eggs were ready to hatch. Newly hatched larvae were exposed to a range of $[Cu^{2+}]$ with metal-nitrotriacetic acid (NTA) buffer systems in sea water (Sunda *et al.*, 1978). The larvae were cultured in the laboratory until they molted to the megalopa stage as described by Costlow *et al.* (1966). Buffered sea water was changed daily. There were 10 larvae per culture bowl and five replicates for each treatment (Sanders *et al.*, 1983).

The metal-NTA buffer system was made up in 35 parts per thousand sea water which had previously been diluted with distilled water to 20 parts per thousand and contained 10^{-4} *M* NTA, 4.6×10^{-8} *M* $ZnCl_2$, 2.4×10^{-8} *M* $CoCl_2$, 1.0×10^{-8} *M* $MnCl_2$, 1.0×10^{-7} $FeCl_3$, and 1.0×10^{-9} *M* Na_2MoO_4. NaOH was added at 4×10^{-4} *M* to adjust the pH to 8.0 ± 0.1. Copper was added as Cu-NTA to achieve concentrations of 1.8×10^{-8} *M* to 5.7×10^{-4} *M*. Free ion concentrations for Cu^{2+} were computed from metal ion-NTA equilibria (Sunda *et al.*, 1978). These concentrations were based on added metal and ranged from $10^{-12.4}$ *M* to $10^{-7.9}$ *M*.

After they molted to the megalopa stage the larvae were sampled, rinsed with distilled water, and lyophilized immediately. Survival and length of time to megalopa were determined as described by Costlow *et al.*, 1966. The freeze-dried megalopa were weighed to the nearest 0.1 μg (Sanders *et al.*, 1984).

Replicate samples were pooled, rehydrated, and homogenized in 0.02 *M* tris-HCl (pH 7.4) with an acid-washed Teflon pestle tissue grinder. The homogenate was centrifuged at $100{,}000 \times g$, and the resulting supernatant was filtered by centrifugation through a 0.2 μm nylon filter. An aliquot (100 μl) of this filtered cytosol was chromatographed on a Toyo Soda steric exclusion (SEC) HPLC column (TSK SW 3000; Sanders *et al.*, 1983). Copper concentrations in the eluant fractions were determined by graphite furnace atomic absorption spectrophotometry (Sanders *et al.*, 1983). Chromatographic data were analyzed by correlation analysis, linear and orthogonal polynomial regression analysis, and analysis of variance (Sokal and Rohlf, 1981). Our confidence limits were set at 0.05.

Results

Cytosolic Cu was associated with three major pools of ligands (Fig. 1): (1) high molecular weight ligands (HMW; ⩾20,000 daltons), the majority of which elute with or just behind the void volume; (2) a metallothionein peak (MT) which has an

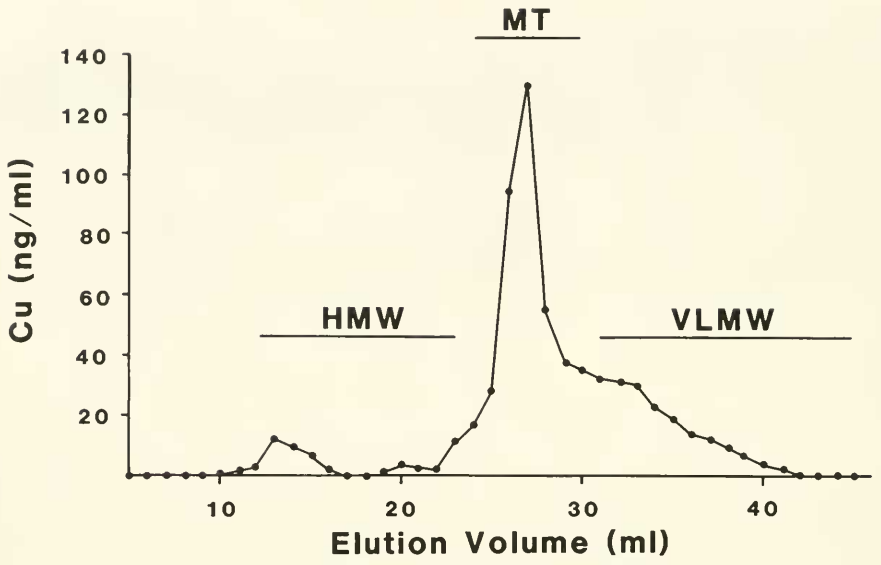

FIGURE 1. Cytosolic distribution of copper in larvae of the crab *Rhithropanopeus harrisii* exposed to free cupric ion concentrations in sea water. HMW, MT, and VLMW represent high molecular weight, metallothionein, and very low molecular weight pools, respectively. The bars represent fractions which were combined for each pool.

apparent molecular weight of 10,000 daltons, and (3) very low molecular weight ligands (VLMW; <5000 daltons) which included molecules too small to be resolved by the column.

There was a biphasic relationship between $[Cu^{2+}]$ in sea water and Cu-thionein and LMW Cu. At $[Cu^{2+}]$ from $10^{-12.68}$ to $10^{-10.6}$ *M* copper metabolism appeared independent of external $[Cu^{2+}]$ (Fig. 2a). At these concentrations there was no significant correlation between external $[Cu^{2+}]$ and Cu-thionein. Copper associated with the VLMW pool was not detectable at environmental concentrations below $10^{-11.0}$ *M*. At concentrations greater than $10^{-10.6}$ *M*, however, Cu in the metallothionein and the VLMW pools correlated significantly with increases in $[Cu^{2+}]$ in sea water ($F = 220$; d.f. = 1, 2; $F = 365$; d.f. = 1, 2). This copper accumulation was reflected in total cytosolic Cu ($F = 363$; d.f. = 1, 2).

The relationship between Cu associated with the HMW ligands and $[Cu^{2+}]$ in sea water contrasted with those of the other pools (Fig. 2b). Although Cu associated with these ligands was significantly above background ($F = 1096$; d.f. = 8, 36), it did not correlate with $[Cu^{2+}]$ in sea water and no accumulation phase was observed (Fig. 2b).

We examined the relationships between $[Cu^{2+}]$ in sea water, survival, duration of the zoeal stages, and megalopa weight to determine the physiological affect of changes in intracellular Cu distribution. Free cupric ion concentrations did not correlate with survival or the duration of zoeal development at concentrations less than $10^{-8.9}$ *M*. At the two highest concentrations tested ($10^{-7.9}$ *M* and $10^{-8.4}$ *M*), all larvae died within three days of exposure. Megalopa weight did correlate with $[Cu^{2+}]$ with significant linear ($F = 53$; d.f. = 1, 36) and quadratic components (Fig. 2c; $F = 50$; d.f. = 1, 36). Megalopa weight was greatest for larvae exposed to $10^{-11.0}$ *M* $[Cu^{2+}]$ and decreased rapidly at higher concentrations.

Copper in the MT and VLMW ligand pools correlated with megalopa weight (Fig. 3; $r^2 = 0.676$; $r^2 = 0.826$). Megalopa weight increased with increases in Cu-

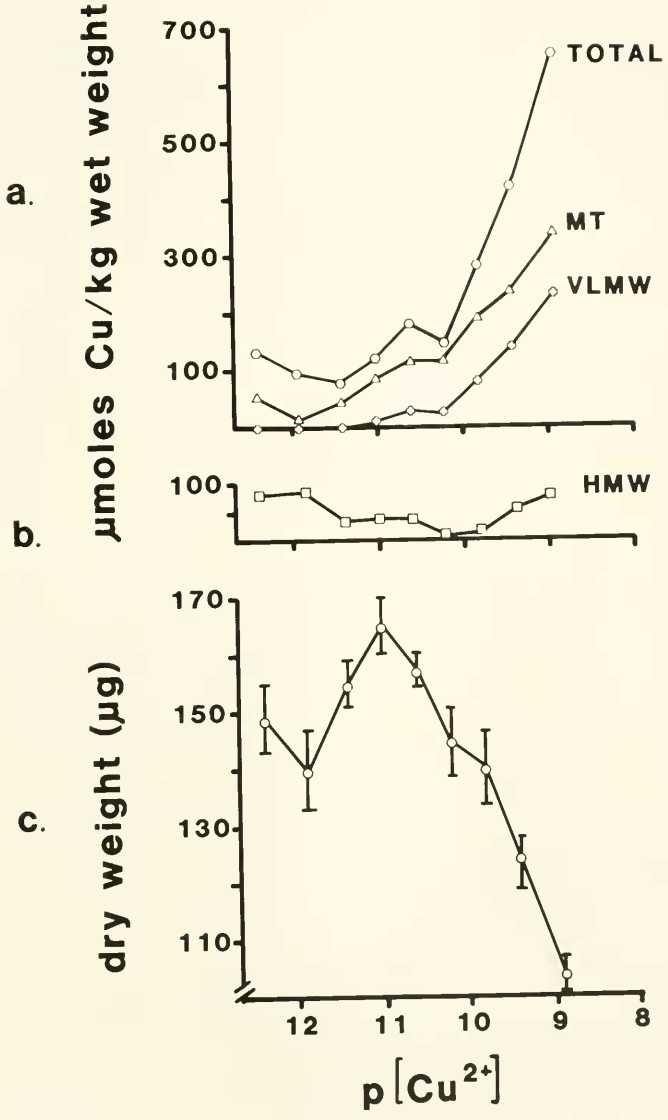

FIGURE 2. (a) Cytosolic distribution of copper expressed in micromoles per kilogram (wet weight) of tissue in *R. harrisii* megalopa exposed to a range of values of $p[Cu^{2+}]$ ($-\log$ of free cupric ion concentration) throughout the duration of larval development. Total, MT, and LMW represent total cytosolic copper, metallothionein, and very low molecular weight ligand pools, respectively. (b) As described above for high molecular weight (HMW) ligand pool. (c) Dry weights of megalopa described above. The vertical lines are one standard error of the mean for five replicates.

thionein until a concentration of 72.6 μm Cu kg^{-1} wet weight ($10^{-11.0}$ *M* exposure), and decreased rapidly at higher concentrations. Although Cu was undetectable in the VLMW pool at the three lowest $[Cu^{2+}]$, it accumulated at higher concentrations. Megalopa weight decreased rapidly with increases of Cu in this pool. In contrast to the MT and VLMW ligand pools, Cu in the HMW pool did not correlate with weight over the entire range of exposures.

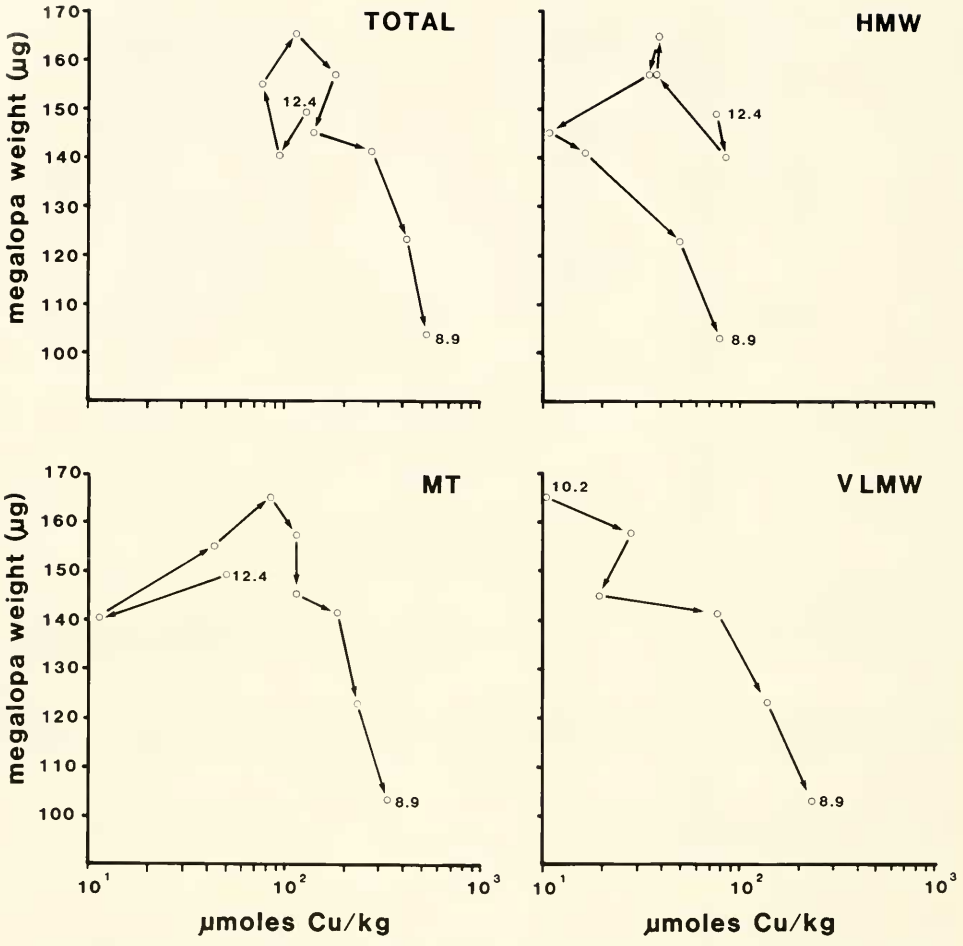

FIGURE 3. Vector diagrams of megalopa dry weight and copper associated with the total cytosol and each cytosolic ligand pool (expressed as micromoles per kilogram dry weight of tissue) in *R. harrisii* megalopa. Total, HMW, MT, and VLMW represent total cytosolic copper, high molecular weight, metallothionein, and very low molecular weight ligands pools, respectively. The arrows delineate the $p[Cu^{2+}]$ (−log free cupric ion concentration) to which the larvae were exposed throughout development from the lowest, to the highest exposures ($p[Cu^{2+}]$ from 12.4 to 8.9).

DISCUSSION

Cytosolic copper accumulation and distribution

The cytosolic distributions of Cu among the HMW, MT, and VLMW ligand pools were similar to those previously reported for *R. harrisii* (Fig. 1; Sanders *et al.*, 1983) and for other crab species (Olafson *et al.*, 1979b; Overnell, 1982). These three metal binding pools have also been reported in other marine invertebrates (Coombs, 1974; Howard and Nickless, 1977, 1978; Jenkins *et al.*, 1982; Frazier and George, 1983).

In our fractionation procedure the HMW pool contained a heterogeneous group of Cu ligands. Most of these ligands are soluble proteins. A number of these proteins, including Cu metalloenzymes and the respiratory pigment hemocyanin, have specific

Cu requirements (Brouwer *et al.*, 1983). Nonspecific binding of Cu to macromolecules in this pool can also occur.

The chemical characterization of metallothioneins in crabs includes amino acid analysis in two species (Olafson *et al.*, 1979a; Overnell, 1982) and amino acid sequencing for one species (Lerch *et al.*, 1981, 1982). Characterization of the MT pool of *R. harrisii* was described in our previous study (Sanders *et al.*, 1983). As with other crab (Olafson *et al.*, 1979a; Overnell and Trewhella, 1979) and mammalian (Kagi and Nordberg, 1979) metallothioneins, it resolves as two major metal binding ligands when fractionated by ion exchange chromatography.

The chemical identity and origin of the VLMW ligand pool is not known but it appears to be made up of a heterogeneous group of Cu complexes. Amino acids may represent an important component of this pool, but these data have yet to be confirmed (Coombs, 1974; Howard and Nickless, 1977, 1978). Frazier and George (1983) have suggested that *in vivo* these VLMW ligands may be associated with granules which become solubilized during homogenization and extraction. Granules containing high concentrations of Cu are present in the hepatopancreas of another crustacean (Weiser and Klima, 1969) but it is not clear if this is the source of the VLMW ligands in crab larvae.

Copper regulation and metabolism

The relationship between $[Cu^{2+}]$ in sea water and cytosolic Cu fell into two discrete patterns (Fig. 2). This biphasic response can be related to the ambient $[Cu^{2+}]$ ($\leq 10^{-10.4}$ *M*) estimated by Sunda and Gillespie (1979) for estuarine waters where the crabs were collected. The first phase occurred at a range of $[Cu^{2+}]$ which is equal to or less than the estimated ambient concentration ($10^{-12.4}$ *M* to $10^{-10.6}$ *M*). In this range of 1.8 orders of magnitude, Cu in the HMW, MT, and VLMW ligand pools were independent of $[Cu^{2+}]$. These data support our previous observations (Sanders *et al.*, 1983) and indicate that Cu accumulation and distribution among the three ligand pools is precisely regulated in crab larvae at exposures at or below ambient $[Cu^{2+}]$.

When larvae are exposed to $[Cu^{2+}]$ which are greater than the ambient concentration ($10^{-10.2}$ *M* to $10^{-8.9}$ *M*), a second phase, one of accumulation, was observed (Fig. 2a). In this phase total cytosolic Cu increased with increases in $[Cu^{2+}]$ in sea water. This accumulation was the result of increases in both Cu-thionein and Cu in the VLMW ligand pool. Accumulation of total cytosolic Cu and Cu-thionein in *R. harrisii* larvae exposed to greater than ambient $[Cu^{2+}]$ has been reported previously (Sanders *et al.*, 1983). In that study cytosolic Cu accumulation and Cu-thionein concentration approached apparent saturation at $[Cu^{2+}]$ just below those which resulted in larval death. This saturation, however, was based on a minimum of data points. In contrast, the data presented here showed no evidence of saturation and the concentrations of total Cu and Cu-thionein attained prior to larval mortality were several fold higher than in the previous experiment (Fig. 3a).

In marked contrast to the other ligand pools, Cu in the HMW ligand pool did not correlate with $[Cu^{2+}]$ in sea water (Fig. 2b). The lack of Cu accumulation in this pool suggests that crab larvae can regulate subcellular Cu distribution over the 3.5 orders of magnitude range of $[Cu^{2+}]$ used in this experiment. Also, the simultaneous increase of Cu in the MT and VLMW ligand pools suggests that they may function as Cu reservoirs which limit nonspecific binding to the HMW ligands.

Free cupric ion concentration and larval growth

In order to understand the ecological significance of Cu accumulation and cytosolic distribution, we examined three parameters: survival, duration of zoeal development,

and megalopa weight. However, only changes in megalopa weight correlated with $[Cu^{2+}]$ (Fig. 2c). Maximum megalopa weight occurred within the range of the estimated ambient $[Cu^{2+}]$ and decreased rapidly at concentrations beyond this range. This pattern of a hormetic overshoot in weight followed by a rapid decrease is an inherent characteristic of stress etiology which has been reported previously in *R. harrisii* larvae (Laughlin *et al.,* 1981; Sanders *et al.,* 1983; 1984). These growth data support our previous observation that fluctuations in ambient $[Cu^{2+}]$ in sea water could limit crab larval growth (Sanders *et al.,* 1983). Cupric ion concentrations near the ambient range also limit growth and nutrient uptake in phytoplankton and bacteria (Jackson and Morgan, 1978; Fitzwater *et al.,* 1982; Sunda and Ferguson, 1983; Jenkins *et al.,* 1984). Thus, natural variations in $[Cu^{2+}]$ could regulate productivity in a wide range of estuarine and marine species through the inhibition of important metabolic processes. The effects of this inhibition could, in turn, modify population dynamics and community composition (Sunda *et al.,* 1981).

Copper accumulation and larval growth

It has been suggested that as long as metals can be sequestered in the MT pool the organism is protected against the toxicity of these metals (Brown *et al.,* 1977, 1983; Jenkins *et al.,* 1982; Nolan and Duke, 1983). However, our data indicate that when larvae were exposed to $[Cu^{2+}]$ beyond the ambient range, the accumulation of Cu in the MT pool was correlated with growth inhibition (Fig. 3).

Although the nonspecific binding of metals to HMW ligands is proposed to be a major mechanism of metal toxicity (Winge *et al.,* 1973; Brown, 1977; Pruell and Engelhardt, 1980), our data show no correlation between Cu in the HMW ligand pool and toxic effects on growth (Fig. 3). Several explanations may account for this apparent discrepency: (1) the techniques used in this study can only detect changes in total Cu and, therefore, do not necessarily distinguish changes in the level of nonspecific binding; (2) the mechanism of metal toxicity may involve the nonspecific binding of Cu to membrane-bound metalloenzymes or macromolecules in other subcellular compartments; and (3) finally Cu uptake and accumulation may modify the uptake and metabolism of other essential metals (*e.g.,* Zn) which may in turn affect larval growth (Bremner and Campbell, 1980; Sunda *et al.,* 1981; Cousins, 1983).

Of the three cytosolic ligand pools, it was the accumulation of Cu in the VLMW pool which was most indicative of toxic effects on the organism. The detection of Cu in this pool was concomitant with perturbations of larval growth. As the Cu concentration in the VLMW pool increased, larval growth rapidly decreased. Cadmium accumulation has been reported in the VLMW pool of the oyster (*Ostrea edulis*) during chronic Cd exposure (Frazier and George, 1983). Although the authors suggest that sequestering cadmium in this pool may protect the oyster from Cd toxicity, they provide no information on the relationship between this accumulation and sublethal effects at the organismic level.

These data emphasize the importance of understanding the interactions among all cytosolic ligand pools. They also underscore the need to correlate these interactions with sensitive indicators of stress (such as growth) before the toxic effects of metals on an organism can be estimated from data on subcellular metal distributions. Finally, these data indicate that changes in intracellular metal metabolism and growth in crab larvae occur at the upper range of the ambient $[Cu^{2+}]$ in the estuary, suggesting that $[Cu^{2+}]$ in sea water may limit larval growth.

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